PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q88296
Gianfranco GILARDI	
Appln. No.: 10/537,612	Group Art Unit: 1652
Confirmation No.: 4772	Examiner: Mohammad Y. MEAH
Filed: January 26, 2006	

For: ENGINEERING REDOX PROTEINS

RESPONSE TO RESTRICTION REQUIREMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This responds to the Restriction Requirement, dated January 17, 2007, time for responding extended to April 17, 2007, by a Petition For Extension Of Time Under 37 C.F.R. §1.136, filed concurrently herewith.

In response to the Restriction Requirement, Applicants elect Group IV, claims 1-5, 12-13, 14-15, 21-22, and SEQ ID NO:11 for examination. This election is made with traverse.

Applicants' respectfully traverse the restriction. On page 4 of the Office Action dated January 17, 2007 the Office asserts that Groups I-IV and IX-XII share a protein (4-alpha helix bundle motif ROP protein) but ignores the presence of the redox center recited in the claims. It is apparent, *i.e.,* at least at the abstract, claims as filed, paragraphs 8-15 and the teachings of Publication No. 20060148026, that a redox center is coupled to a repressor of primer. The invention includes a special technical feature which provides a contribution over the cited reference since Predki and Regan only disclose four-helix-bundle proteins. Under P.C.T. Rule 13.2, Predki and Regan cannot serve as an anticipatory reference since the cited reference does not prove that Applicants' invention is not so linked as to form a general inventive concept and since the reference fails to disclose Applicants' contribution over the art.

To better illustrate the special technical feature, Applicants herewith submit an article by Castagnoli *et al.* that provides a genetic and structural analysis of ROP. The genetic sequence referred to in Castagnoli *et al.* is found in Cesareni *et al.*, a copy of which is also enclosed. Wild type ROP is a dimeric protein - each monomer is formed by two helices connected by a sharp bend. The wild type sequence is disclosed by Cesareni *et al.* and is included in the top row of the enclosed sequence alignment. For ease of analysis, only amino acid sequences are provided.

As the sequence alignment of Appendix A clearly shows, SEQ ID NO:2 of the present Application is identical to helix 1 of the wild type sequence. Similarly, SEQ ID NO:4 of the present application is identical to helix 2 of the wild type sequence. SEQ ID NO:6 of the present application represents one half of the wild type dimeric ROP, namely helices 1 and 2. As the sequence alignment also shows, SEQ ID NO:6 is identical to the wild type protein sequence found in Cesareni *et al.*

SEQ ID NO:8 is a monomeric ROP having all four helices expressed as a single polypeptide chain. As set out on page 1, lines 21 to 27 of PCT/GB03/05256, "Predki and Regan (1995) describe producing a single chain rop. Their method separates helix 1 and 2 sequences of one rop molecule and places helix 1 before helix 1′ and places helix 2 and helix 2′ of a second rop (consisting of helices 1′ and 2′). The monomeric rop contains 4 a-helices in the order 1-1′-2′-2 and all a helices are expressed as a single polypeptide chain". As observed in the sequence alignment, helices 1, 2, 1′ and 2′ are identical to those found in the wild type sequence of Cesareni *et al.*

SEQ ID NO:11 is based on the Predki and Regan monomeric sequence and includes five point mutations. SEQ ID NO:11 provides an example of point mutations that can be made to enhance the binding interaction of ROP with a redox group. The mutations clearly fall within typical confidence limits when assessing identity of amino acid sequences. Indeed, SEQ ID NO:11 is 96% identical to that of SEQ ID NO:8 and thus, by inference, with the wild type sequences set out in SEQ ID NOS:2, 4 and 6 and as described in Cesareni *et al.*

Based on these similarities, Applicants assert that Groups I-IV should be searched and examined together.

According to the Office, invention Groups V to VIII relate to claims drawn to a DNA that encodes a polypeptide, vector, transformant and method of production of the proteins of respective Groups I to IV. For the reasons set out above, Applicants assert that all DNA sequences should be searched and examined as one invention. Indeed, since the amino acid sequences are derived from the DNA sequences, Groups I to VIII relate to the same inventive concept and so should be searched and examined simultaneously. The reasons set out for Groups I to IV, V to VIII and I to VIII also apply to invention Groups IX to XII. Accordingly, Applicants assert that the claims relate to one inventive concept, share a technical feature and therefore should be searched and examined together.

Applicant reserves the right to file a Divisional Application directed to non-elected claims.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

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Date: April 17, 2007

APPENDIX A

Cesareni et al: Met ..... Thr Lys Gln Glu Lys Thr Ala Leu Asn Met Ala Arg Phe Ile Arg Ser Gln Thr Leu Thr Leu Leu Glu Lys Leu Asn Glu Leu
Seq ID No.2: ..... Thr Lys Gln Glu Lys Thr Ala Leu Asn Met Ala Arg Phe Ile Arg Ser Gln Thr Leu Thr Leu Leu Glu Lys Leu Asn Glu Leu
Seq ID No.4: .....
Seq ID No.6: Met Gly Thr Lys Gln Glu Lys Thr Ala Leu Asn Met Ala Arg Phe Ile Arg Ser Gln Thr Leu Thr Leu Leu Glu Lys Leu Asn Glu Leu
Seq ID No.8 Met Gly Thr Lys Gln Glu Lys Thr Ala Leu Asn Met Ala Arg Phe Ile Arg Ser Gln Thr Leu Thr Leu Leu Glu Lys Leu Asn Glu Leu
Seq ID No.11 Met Gly Thr Lys Gln Glu Lys Thr Ala Leu Asn Met Ala Arg Phe Ile Arg Ser Gln Thr Leu Thr Leu Leu Glu Lys Leu Asn Glu Leu

C .....
2 .....
4 .....
6 .....
8 Gly Gly Gly Gly Gly Thr Lys Gln Glu Lys Thr Ala Leu Asn Met Ala Arg Phe Ile Arg Ser Gln Thr Leu Thr Leu Leu Glu Lys Leu
11 Gly Gly Gly Gly Gly Thr Lys Gln Glu Lys Thr Ala Leu Asn Met Ala Arg Phe Ile Arg Ser Gln Thr Leu Thr His Leu Glu Lys Leu

C .....
2 .....
4 .....
6 .....
8 Asn Glu Leu Gly Ala Asp Glu Gln Ala Asp Ile Cys Glu Ser Leu His Asp His Ala Asp Glu Leu Tyr Arg Ser Cys Leu Ala Arg Phe
11 Asn Glu Leu Gly Ala Asp Glu Gln Ala Asp Ile Cys Glu Ser Leu Ala Asp Trp Ala Asp Glu Leu Tyr Arg Ser Cys Leu Ala Arg Phe

C ..... Asp Ala Asp Glu Gln Ala Asp Ile Cys Glu Ser Leu His Asp His Ala Asp Glu Leu Tyr Arg Ser Cys Leu Ala
2 .....
4 ..... Asp Glu Gln Ala Asp Ile Cys Glu Ser Leu His Asp His Ala Asp Glu Leu Tyr Arg Ser Cys Leu Ala
6 ..... Asp Ala Asp Glu Gln Ala Asp Ile Cys Glu Ser Leu His Asp His Ala Asp Glu Leu Tyr Arg Ser Cys Leu Ala
8 Gly Gly Gly Gly Gly ..... Ala Asp Glu Gln Ala Asp Ile Cys Glu Ser Leu His Asp His Ala Asp Glu Leu Tyr Arg Ser Cys Leu Ala
11 Gly Gly Gly Gly Gly ..... Ala Asp Glu Gln Ala Asp Ile Cys Glu Ser Leu Ala Asp Trp Ala Asp Glu His Tyr Arg Ser Cys Leu Ala

C	Arg Phe Gly Asp Asp Gly Glu Asn Leu
2	.....
4	Arg Phe Gly Asp Asp .....
6	Arg Phe Gly Asp Asp Gly Glu Asn Leu
8	Arg Phe Gly Asp Asp Gly Glu Asn Leu
11	Arg Phe Gly Asp Asp Gly Glu Asn Leu

The EMBO Journal vol.8 no.2 pp.621-629, 1989

Genetic and structural analysis of the ColE1 Rop (Rom) protein

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Communicated by G.Cesareni

Repressor of primer (Rop) is a small dimeric protein that participates in the mechanism that controls the copy number of plasmid of the ColE1 family by increasing the affinity between two complementary RNAs. The Rop dimer is a bundle of four tightly packed α-helices that are held together by hydrophobic interactions. We have systematically altered, by site directed mutagenesis, most of the solvent exposed amino acids of the Rop bundle and we have identified the alterations that cause a decrease of the activity of the regulatory molecule. We conclude that Rop folding is rather insensitive to amino acid substitutions and to other mutations as drastic as deletions and insertions. Looking along the 2-fold symmetry axis the amino acid side chains whose alterations affect the function of Rop are all located on one side of the molecule. Furthermore they are clustered at the extremities of the α-helix bundle, the only exception being the aromatic ring of Phe-14.

Key words: ColE1/copy number/mutagenesis/Rop (Rom) protein

Introduction

Multicopy plasmids, derived from plasmids of the ColE1 family, have evolved an efficient regulation mechanism that helps to maintain a constant copy number by counteracting occasional deviations from the steady state level (For a review see Cesareni and Banner, 1985). This is achieved by negative control of the frequency of replication initiation events, mediated by the interaction of two RNA molecules, RNAI and RNAII, and a protein of 63 amino acids, repressor of primer (Rop). RNAII is transcribed starting from a promoter 550 nt upstream from the replication origin and in the proximity of the origin forms a persistent hybrid with the DNA template. The factors that determine the formation of this hybrid are not completely understood. This process however has been shown to be influenced by secondary structure elements that extend over most of the RNA molecule (Masukata and Tomizawa, 1984, 1986). The formation of the hybrid at the origin is essential to promote initiation of DNA replication since the RNA part of the hybrid is digested by RNase H to yield a molecule of 550 nt that can be used by DNA polymerase 1 as a primer for initiation of DNA synthesis (Itoh and Tomizawa, 1980).

RNAI and Rop act negatively in this regulation mechanism, as shown by the elevated copy number of plasmids defective in these functions (Conrad and Campbell, 1979; Twigg and Sherratt, 1980). RNAI is a molecule of 110 nt encoded by the strand opposite to the one that directs the synthesis of the 5′ end of RNAII. Genetic and biochemical analysis has shown that RNAI exerts its regulatory function by hydrogen bonding to the complementary sequence in RNAII, the precursor of the primer (Lacatena and Cesareni, 1981, 1983; Tomizawa and Itoh, 1981). This interaction prevents RNAII from folding into a secondary structure that promotes the formation of the persistent hybrid at the origin and thus inhibits RNase H processing and, as a consequence, primer formation and initiation of DNA replication (Mazukata and Tomizawa, 1986). The kinetics of the interaction between RNAI and the nascent RNAII have been studied in great detail by Tomizawa (1984) who showed that the process occurs in two steps. The first step is faster and reversible and involves the hydrogen bonding of some nucleotides in the three complementary loops. The end product, however, is a complete hybrid between the two RNAs.

The second inhibitor of plasmid replication has been identified as a protein of 63 amino acids (Cesareni *et al.*, 1982). The gene encoding it has been named Rop (repressor of primer) since it was observed that it can negatively regulate the expression of genes under the control of the primer promoter (Cesareni *et al.*, 1982; Som and Tomizawa, 1983). *In vivo* and *in vitro* studies however have pointed out that Rop does not act as a classical repressor but enhances the inhibitory activity of RNAI (Cesareni *et al.*, 1984; Lacatena *et al.*, 1984). Tomizawa and Som (1984) have shown that, in the presence of Rop, the hybridization of RNAI with the primer occurs faster than in its absence and, more specifically, that Rop increases the rate of the first reversible step of the interaction. Because of this observation these authors have changed the name of the protein into Rom (RNA One Modulator) to underline its better defined function.

The hybridization mechanism regulated by Rop is not the only molecular mechanism that depends on the hybridization of two RNAs and is regulated by a protein. Recently the initiator protein of the ColE1-unrelated plasmid R6K and the NBP.P12 protein of the avian retrovirus have been shown to have RNA annealing activity (Indravadan and Bastia, 1987; Prats *et al.*, 1988).

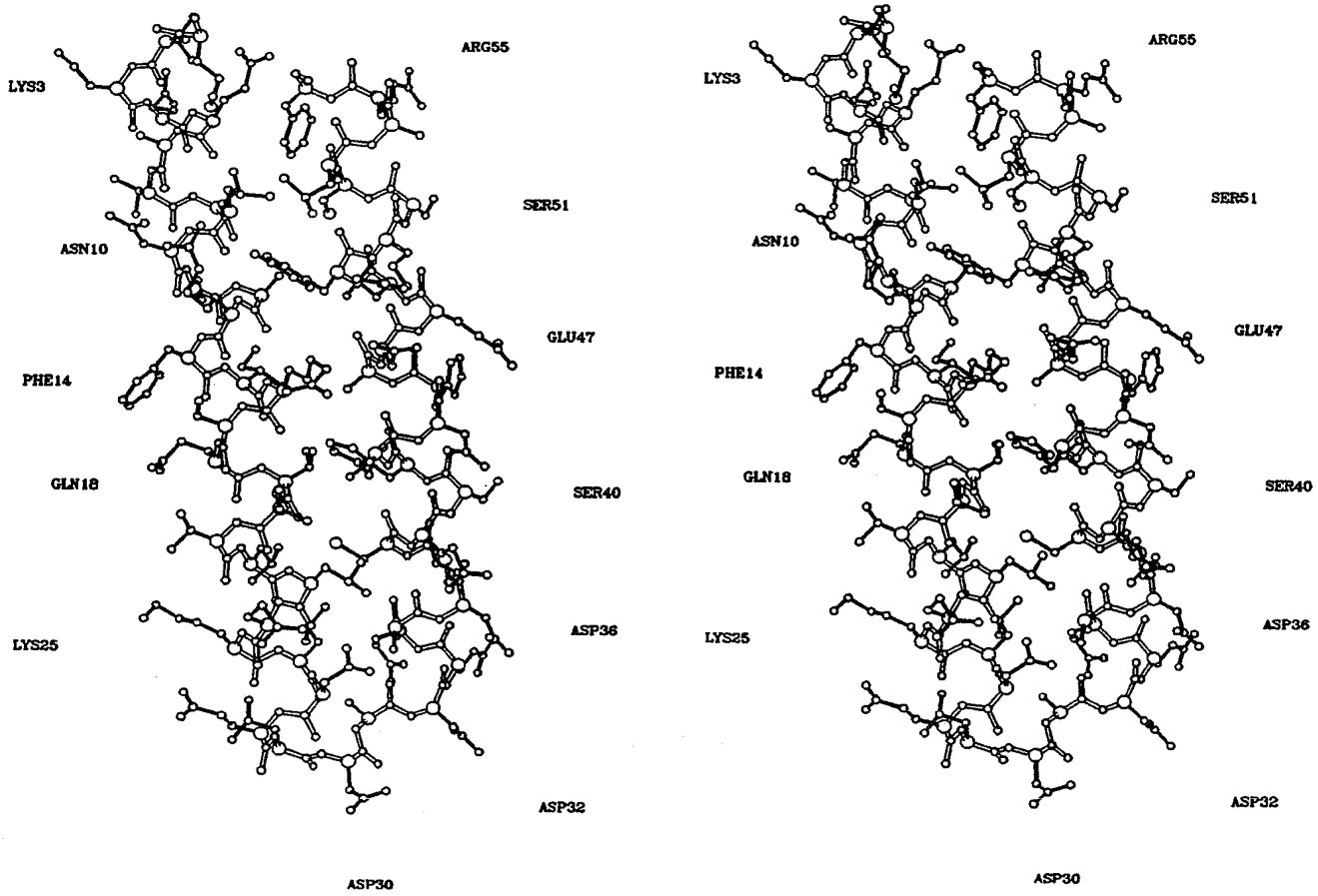


Fig. 1. The structure of Rop. Stereo diagram of the Rop monomer. The monomer is viewed from the outside of the molecule, the 2-fold axis being horizontal behind the molecule. Residue numbers are indicated (Banner *et al.*, 1987).

We have recently shown that Rop specifically interacts with the stem structures of RNAI and RNAII and we have proposed that Rop performs its function by acting as an adaptor for the correct positioning of the two RNA molecules (Helmer-Citterich *et al.*, 1988). Here we discuss the high resolution structure of Rop and we analyse the phenotype of several mutants in which solvent exposed side chains have been altered.

Results and Discussion

The three dimensional structure of Rop

To elucidate the function of Rop at the molecular level we have overexpressed, purified and solved by X-ray crystallography the three-dimensional structure of the protein (Lacatena *et al.*, 1984; Banner *et al.*, 1983, 1987). The native Rop is a dimer as shown by its migration properties in a gel filtration column and by its structure in crystals. Each of the two subunits folds in a very simple and regular structure constituted by two α-helices connected by a sharp bend (Figure 1). The two subunits assemble in the dimer to form a bundle of four α-helices with an exact 2-fold symmetry axis. The resulting molecule is very compact and stable to heat and chemical denaturants (our unpublished results). The forces that bring the four α-helices together are essentially hydrophobic while ionic interactions do not seem to play any role.

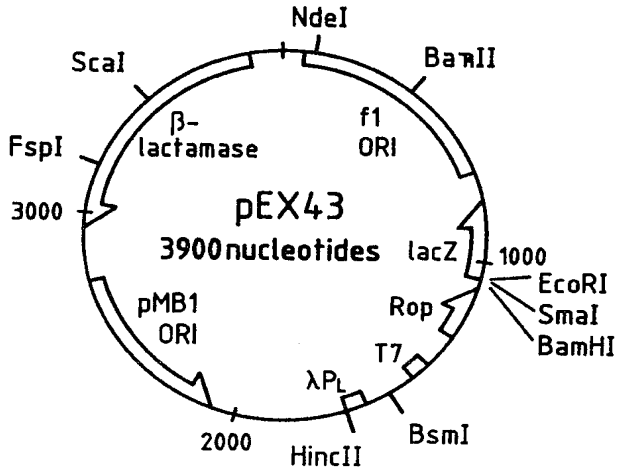


Fig. 2. Map of pEX43. The relevant genetic loci are shown. The construction of the plasmid is described in Materials and methods.

The dimeric and symmetric structure of the Rop molecule and the observation that Rop specifically binds, albeit at low

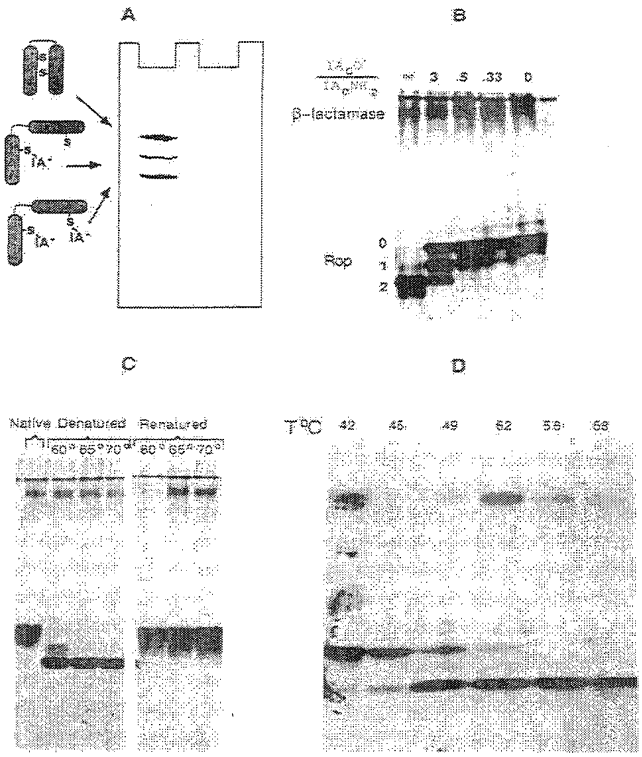


Fig. 3. Test for Rop folding. **Panel A** schematically illustrates the different migration of Rop molecules with one or two Cys carboxylated by iodo-acetic acid with respect to the native unmodified molecule. All the gels are 15% polyacrylamide gels in Tris–glycine buffer containing 6 M urea. The samples (*in vitro* transcription–translation products of plasmid pEX43) were treated in different conditions with iodo-acetic acid (see Materials and methods) and electrophoresed overnight at 50 V on a 0.1 mm thick gel. **Panel B** the five samples were incubated with different concentration ratios (indicated above the gel lanes) of iodo-acetic acid and iodo-acetamide. The latter chemical blocks the thiol groups without changing their charge. The figures on the left of the gel indicate the number of extra carboxyl groups corresponding to each band. **Panel C**, the denaturation of Rop is reversible in this system. The *in vitro* translation products were heated at three different temperatures (60, 65 and 70°C) and then cooled down to 37°C. The seven samples, native control and denatured and renatured protein at the three different temperatures, were all treated for 10 min at 37°C with iodo-acetic acid and then analysed by electrophoresis. **Panel D**, the carboxylation reaction was carried out for 10 min at the temperatures indicated above the gel lanes.

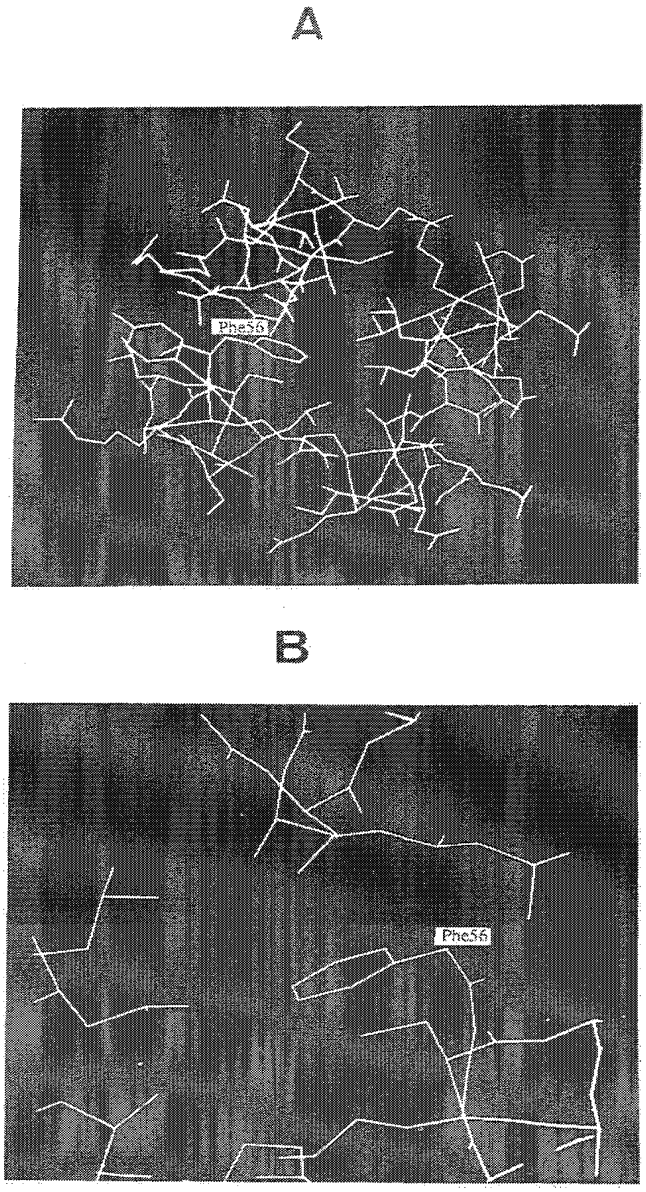


Fig. 4. Two different views of the tip of the Rop bundle that illustrate details of the structure in the proximity of Phe-56.

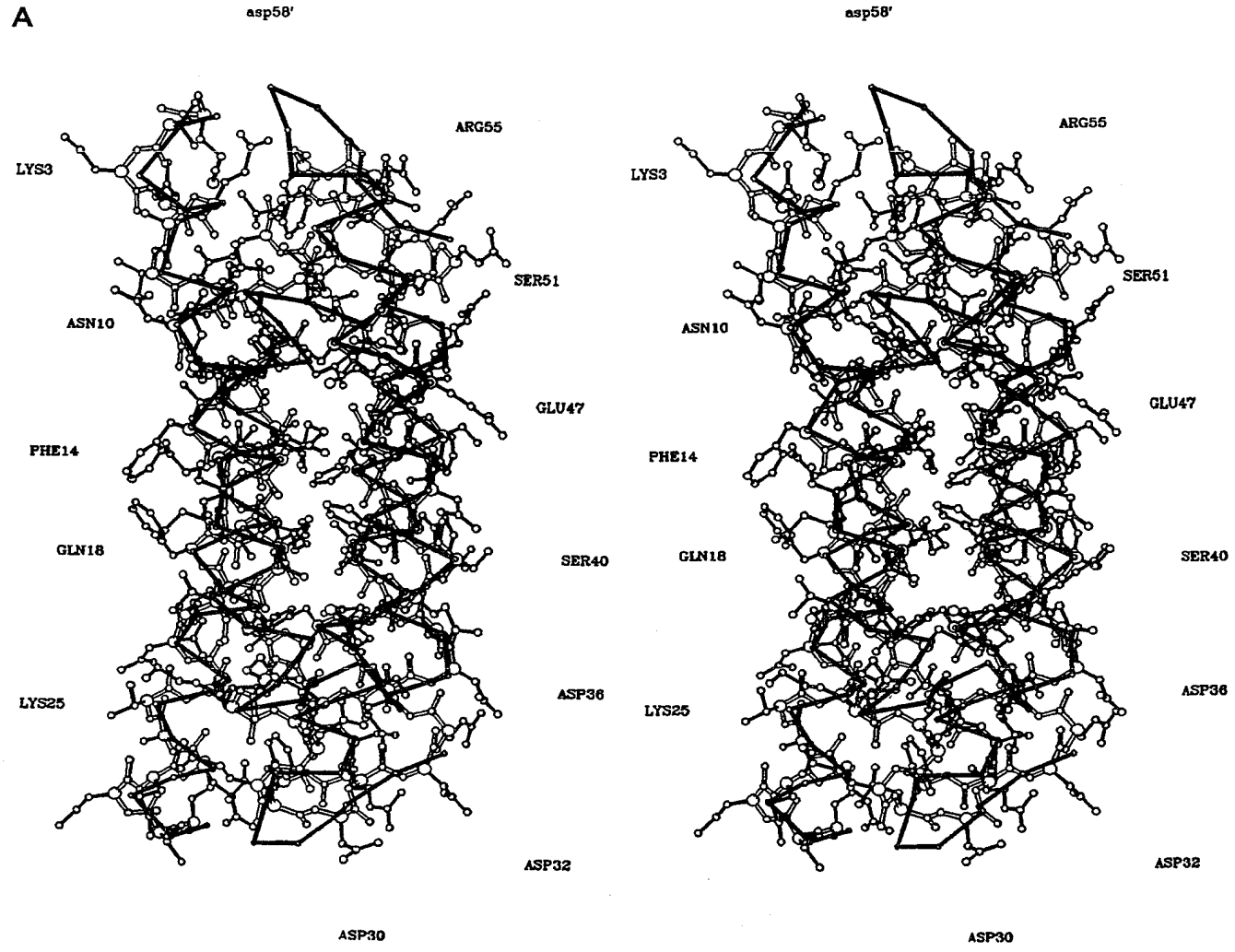
affinity, RNAI and RNAII (Helmer Citterich *et al.*, 1988) lead us to propose a model where each subunit of the dimer recognizes similar ribonucleotide sequences in stem structures and functions as an adaptor that correctly positions the two RNAs for loop interaction (Cesareni and Banner, 1985; Helmer Citterich *et al.*, 1988). To gain further information about the geometry of this interaction and to identify the amino acid side chains that are responsible for RNA recognition, we have systematically modified the amino acids whose side chains are exposed to the solvent and we have determined whether these altered proteins still maintain their function.

For mutant isolation and overexpression we have made use of an expression plasmid (pEX43) (Figure 2) containing the replication origin of phage f1. This allows us to prepare the plasmid chromosome in a single stranded form for oligonucleotide directed mutagenesis and for mutant sequencing (Dente *et al.*, 1983; Sollazzo *et al.*, 1985). pEX43 has the Rop gene under the control of the p_L promoter of phage λ and in the absence of an active λ cI repressor directs the synthesis of large amounts of Rop protein. A further useful feature of this vector consists in the presence, between the p_L promoter and the Rop structural gene, of a strong promoter (ϕ10) for the RNA polymerase of phage T7 (Tabor and Richardson, 1985). We have exploited this feature to transcribe efficiently the Rop gene *in vitro* and to produce small amounts of specifically labelled (radioactively pure) Rop variants in an *Escherichia coli in vitro* translation system. Rop function was assayed in two ways. As a first quick test we exploited the ability

of Rop to decrease the expression of genes transcriptionally fused to the first 110 nt of the primer precursor. L76, an *E.coli* strain containing a fusion between the β-galactosidase structural gene and the initial nucleotides of the primer transcript forms red colonies on MacConkey plates. In the presence of a ColE1 type plasmid encoding an active Rop gene, however, β-galactosidase levels are decreased and the strain forms white colonies (Cesareni *et al.*, 1982; 1984). We used this test as a screening procedure that allowed us to identify colonies harbouring defective proteins by direct inspection of colony colour on the plate. The second test involved estimating plasmid copy number by determining the ampicillin concentration at which the colony forming ability of a strain is decreased by a factor of 10.

Defective Rop proteins could in principle derive either from correctly folded peptides in which an essential amino acid side chain (i.e. for interaction with RNA) has been altered, or from mutant proteins that do not assume the correct three-dimensional conformation. From our experience, most of the mutants (if not all) that belong to this latter class are degraded by *E.coli* proteases. Thus, to characterize further the functionally negative mutants that we have isolated, we tested whether we could recover Rop protein from a cell extract containing an overproducer plasmid directing the synthesis of the defective protein. Mutant Rop proteins that could be overproduced were assumed to be correctly folded and provisionally classified as 'functional' mutants, while mutants that could not be recovered from *E.coli* extracts were classified as 'folding' mutants. In all the cases in which this assumption was tested by biochemical, spectroscopic or crystallographic techniques it proved to be correct.

As a second, more direct test for Rop folding we devised a method that allowed us to probe rapidly some characteristics of Rop structure without resorting to protein purification. This method relies on the synthesis of small amounts of isotopically pure Rop mutants in an *in vitro* transcription translation system and measures the accessibility of cysteines to thiol modifying chemicals. In the native structure the two cysteines at position 38 and 52 are not exposed to the solvent and are therefore insensitive to carboxylation by iodo-acetic acid. Carboxylation can be monitored on a native gel, since the addition of the carboxyl group changes the mobility of the protein by changing its charge. As shown in Figure 3 Rop is practically insensitive to carboxylation at 42°C. When the temperature is raised



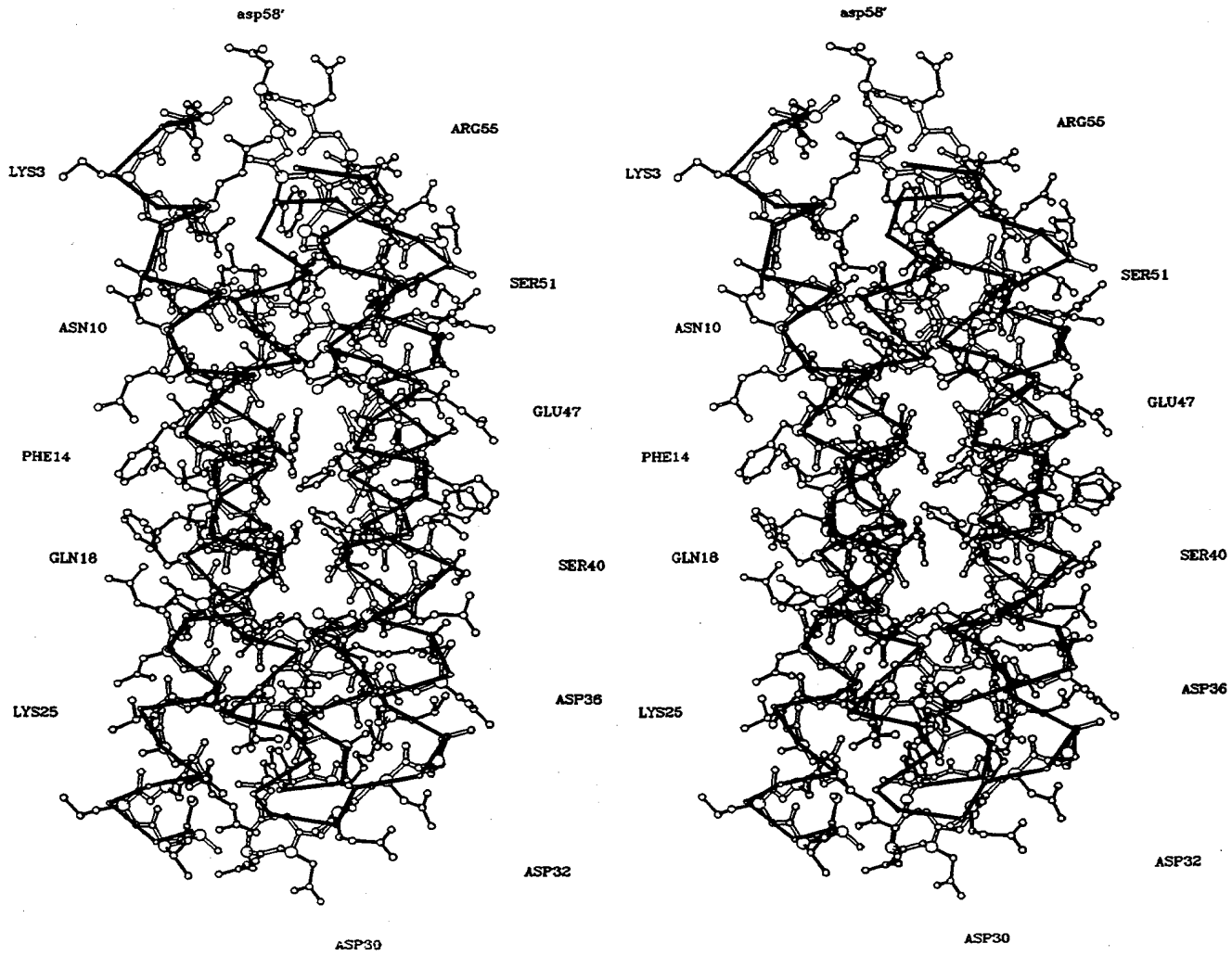


Fig. 5. Comparison of the structures of the dimer of wild type Rop and of its derivative Δ[Asp-30−Gln-34]. In **Panel A** we have superimposed the α-carbon backbone (heavy line) of the mutant onto the structure of the wild type. In **Panel B** the complete structure of the mutant dimer is compared to the α-carbon backbone of the wild type. Only one of the two monomers is labelled with residue numbers. In the mutant structure we have maintained the wild type amino acid numbering.

to 56°C however, the majority of the molecules have both cysteines modified within 10 min (Figure 3).

Deletion and insertion derivatives of Rop

To explore which regions of the Rop dimer could be important for function we have isolated, by site directed mutagenesis, both deletion and insertion mutants. First we tested whether the last seven carboxy-terminal amino acids that are disordered in the crystal and could not be identified unambiguously in the electron density map, are important for function. Thus we isolated two amber mutants, one at position Phe-56 and the second at position Asp-58. These two mutated genes encode, in su^- strains, truncated Rop proteins in which the last eight and six amino acids are missing. As shown in the table in Figure 6, Asp-58-am is still active while Phe-56-am has completely lost activity. These results prove that the six carboxy-terminal amino acids of Rop do not play any role in control of plasmid copy number while either Phe-56 or Gly-57 or both are essential. However, we have never been able to overproduce Phe-56-am protein either *in vivo* or *in vitro*, a result that suggests that one of these two amino acids or both play an important structural role. Pulse and chase experiments demonstrate that this protein is highly unstable. This finding can be retrospectively rationalized by observing that, in the three-dimensional structure, Phe-56 acts as a cork to plug a hydrophobic hole at the base of the Rop cylinder (Figure 4). It is conceivable that by exposing these hydrophobic pockets to the solvent the structure would be destabilized. This conclusion is reinforced by the observation that when the Phe-56-am mutation is suppressed in a *supE* strain that inserts a Gln in place of the amber triplet, the phenotype of the resulting Rop protein becomes temperature sensitive (active at 30°C and inactive at 42°C).

The second Rop structure that could be modified without altering the general folding pattern of the molecule is the

mutation	red/white	Relative copy number (+/- 0.2)	overprod.	carboxylation test	1
Insertion &deletions					
Phe56-am	R	2	N		
Asp58-am	W	1	Y		
Asp32-oc (TAA)	R	1.9			
<2aa>	P	1.3	Y	+	
Δ[Asp30-Gln34]	R	2	Y	+	
Loop region					
Asn27-Ala	W/P	1.3			
Glu28-Ala	W/P	1.3	Y		
Ala31-Pro	P	1.4	Y		
Ala31-Leu	W	1			
Asp32-Phe	R	1.6	Y	+	
Asp32-Trp	W	1.1			
Asp32-Glu	W	1			
Glu33-Ala	W	1			
Asp36-Val	W	1			
Basic amino acids					
Lys3-Cys	W/P	1.5		+	
Lys6-Cys	W	1.3			
Arg13-Cys	W	1	Y	+	
Arg16-Cys	W	0.9	Y		
Arg55-Cys	W	1.2			
Others					
Glu5-Ala	W	1.1			
Phe14-Cys	R	2.2	Y	+	
Glu24-Ala	W	1			
Glu39-Ala	W	1	Y		
Glu47-Ala	W	1			
Asp59-His	W	1	Y		
Leu41-asn	P		N		

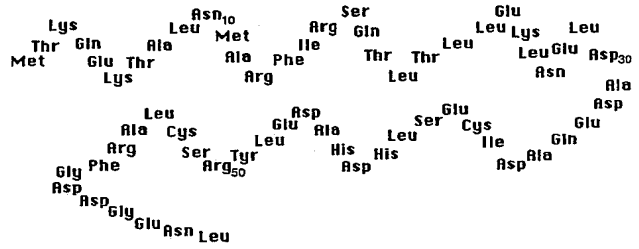


Fig. 6. Sequence of Rop and properties of the mutants that have been isolated. The lower part of the figure is a diagram of the amino acid sequence of Rop including the last seven amino acids that are disordered in the crystal structure. In the top part of the figure we have reported the characterization of the mutants that have been isolated. For the red/white test (see text and Materials and methods for details) the results are reported in column 1 in a semi-quantitative manner. The mutants have been divided into four classes. R indicates that the colony colour was clearly red after 24 h incubation at 37°C. If, after this time, the colour of the colony was pink the corresponding mutant was labelled with P. Mutants that were clearly white after 24 h at 37°C could be further divided into two classes depending on whether the colonies remained white (W) or turned pink (W/P) after further incubation at room temperature for 24 h. The figures in the second column are the relative copy numbers of the plasmid synthesizing the mutant Rop. These figures are averages of three different experiments and were determined by measuring the ampicillin concentration at which the colony forming ability of a given mutant was decreased by a factor of 10 after plating at 37°C. The mutants that we attempted to overproduce and those that were tested by the carboxylation test (see Materials and methods) are indicated in the third and fourth column respectively. Y and N indicate success (Y) or lack of success (N) in the attempt to overproduce the mutant protein by raising the temperature to 42°C to inactivate the thermosensitive allele of the λ repressor that controls Rop expression in plasmid pEX43. In the last column + indicates that the hidden cysteines of the labelled mutant protein synthesized in the *in vitro* system could not be modified by iodo-acetic acid after 5 min incubation in the conditions described in the Materials and methods section. Unlike all the other mutations in the table, Leu-41→Asn does not modify a solvent-exposed side chain. This mutant was included as a negative control for the carboxylation test.

bend region. Here we constructed Δ[Asp-30→Gln-34] where the residues from Asp-30→Gln-34 have been deleted and Σ[Ala-30,Ala-32] where two extra alanines are now flanking Asp-30. The observation that Σ[Ala-30,Ala-32] directs the synthesis of a protein that is only marginally less active than the wild type indicates that this insertion does not disturb protein folding and that the residues important for function are not dramatically affected. By contrast Δ[Asp-30→Gln-34] is completely inactive despite the fact that the protein is not degraded and can be recovered at high yields in *E.coli* extracts. To assess whether the only region that is modified in this altered protein is the bend region we have purified, crystallized and solved the structure of Δ[Asp-30→Gln-34] at 1.5 Å resolution. The structure of this mutant will be described in detail elsewhere. While the crystallographic refinement is still in progress, it is clear that the folding and assembly of this mutant molecule is very similar to the native protein (Figure 5). Most of the backbone of the two proteins can be superimposed without major adjustments. Also the majority of the side chains show no significant conformational differences in the two structures, the most striking exception being the long side chains of Arg and Lys. The conformations of the hydrophobic side chains that form the core of the molecule are not altered. The only major adjustment occurs at the bend region and involves the residues from Leu-26 to Leu-29. These residues, which in the native protein belong to the first helix, now make the connection between the helices, forming a novel type of bend. Interestingly the seven carboxy-terminal amino acids, that were disordered and thus invisible in the wild type electron density map, could be traced easily in the mutant map. In the mutant structure both the bend region and the C-terminus are stabilized by inter- and intra-molecular hydrogen bonds.

Point mutants

Analysis of the deletion/insertion mutants that we have just described proves that while the six carboxy-terminal amino acids are not essential some of the amino acids on one or either side of the bend are essential for function. To locate precisely the amino acid(s) playing the essential role and to analyse which of the solvent exposed amino acids on the lateral surface of the Rop cylinder are involved in the interaction with the RNA, we have isolated a number of point mutants, by site directed mutagenesis, and analysed their properties by the functional and structural tests previously described. The mutants that have been isolated are reported in Figure 6 together with their properties. Only seven of the single amino acid substitutions tested result in a mutant Rop protein that is clearly less active, while the phenotypes of the remaining ones are either indistinguishable from the wild type or are marginally less active. This first result is rather unexpected. Our prejudice was that either the structure or the function of Rop should be very sensitive to amino acid changes. This derived from the observation that the three Rop genes that have been sequenced to date reveal a remarkably high frequency of third base silent changes. The ColE1 and pMB1 Rop genes have a different base in 11 positions, none of which results in an amino acid change (Cesareni *et al.*, 1982). The four amino acid substitutions that have been observed in the more distantly related Rop protein of plasmid ColK, i.e. Thr-19→Ala; Glu-33→Asp; Cys-38→Ser; Ser-40→Ala (Archer, 1985) are all rather conservative changes in terms of the structural constraints of the protein (Banner *et al.*, 1987). This strong tendency to conservation during evolution contrasts with our results that indicate that most of the solvent-exposed amino acids

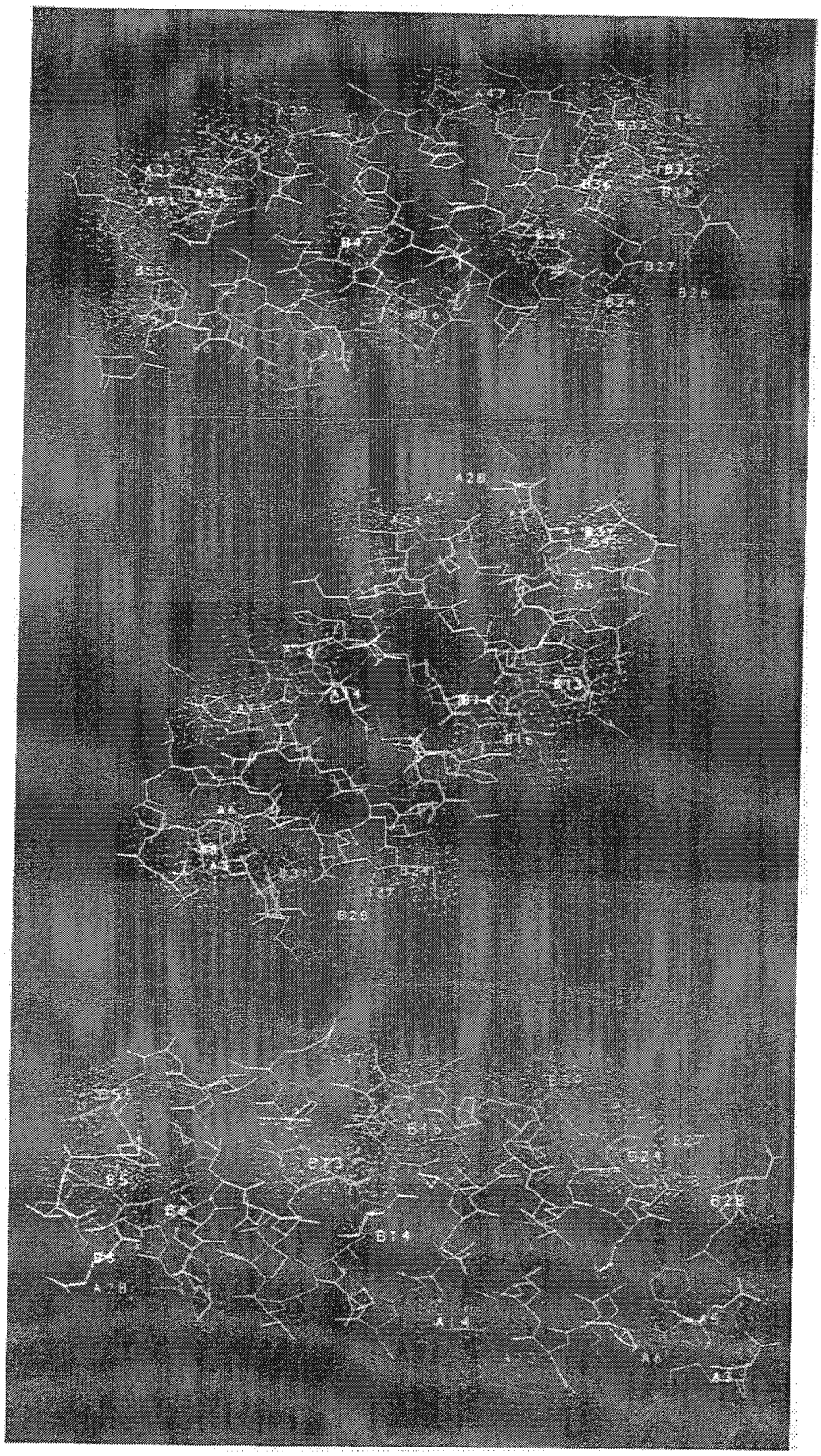


Fig. 7. Localization of active and inactive mutants of the Rop molecule. Three different views of the Rop dimer. The amino acid side chains that have been tested by site directed mutagenesis were identified by their van der Waal surfaces. Side chains whose alteration caused a non-functional protein have red surfaces while the side chains that have been mutated and have not been found to be important for function have blue surfaces. Relevant amino acids have been identified with numbers, indicating their position along the polypeptide chain, preceded either by an A or by a B to distinguish identical residues on the two monomers. The pictures were drawn using the Insight graphic program.

are not important for function as assayed in our two *in vivo* tests and none of them prevents folding. We are forced to conclude that either our assays do not completely cover the function of Rop or that subtle quantitative changes that we cannot detect are, or have been, selectively not neutral in the natural evolutionary niche of these bacterial plasmids.

The loop region

To localize further the essential information that has been affected in the mutant Δ[Asp-30−Gln-34] we have constructed mutants in which the solvent-exposed amino acids from Glu-24 to Asp-36 have been modified by site directed mutagenesis. The only solvent-exposed amino acid that has not been modified is Asp-30. That the role of this amino acid is not critical, however, has already been demonstrated by the construction of mutant Σ[Ala-30,Ala-32] in which two alanines have been inserted on both sides of Asp-30. The mutants at position Asp-32 were obtained by random mutagenesis with an oligonucleotide carrying degenerate sequences in place of the Asp-32 coding triplet. As indicated in the Figure 6 only Phe at position 32 has a drastic effect on function. However when Asp-32 is replaced by Glu or, more surprisingly, by Trp, we do not observe any loss in activity indicating that the function of Asp at position 32 is neither that of providing a negative charge nor a specific pattern of hydrogen bond acceptor or donor groups. As indicated in Figure 6, the defect of Rop (Asp-32−Phe) is not due to problems in folding since the protein synthesized in an *in vitro* system cannot be modified by incubating in the presence of iodo-acetic acid at 37°C.

While Glu-33 and Asp-36 can be modified to Ala or Val without any apparent effect, Asn-27−Ala and Glu-28−Ala result in decreased activity. In the absence of the three dimensional structure of the loop region of these mutants it is impossible to conclude whether these defective phenotypes derive from the substitution of some groups that are important for the interaction with the RNAs or from a less direct effect on loop conformation.

The simple and regular shape of the Rop protein encouraged us to consider the possibility that some portion of the backbone, rather than the specific amino acid side chains, is essential. If this were the case, we could expect that disturbing, for instance, the α-carbon backbone at the bend region should result in an inactive protein. To test this hypothesis we have inserted a Pro in place of Ala-31. This position is not extremely sensitive to substitutions since it is possible to replace the Ala with a Leu without loosing any activity (Figure 6). The dihedral angles of Ala-31 in the wild type molecule are prohibited to Pro. Although we do not have high resolution structure of this mutant yet, thermodynamic and circular dicroism data indicate a gross rearrangement of the structure (H.Hinz and P.Weber personal communication). At 30°C however the mutant protein is only marginally less active than wild type suggesting that, at least in the bend region, main chain hydrogen bonds to the RNA are not essential.

Positively charged side chains

Proteins interacting with nucleic acids have to cope with the highly negatively charged phosphate backbone of the nucleotide chain. It is reasonable to assume that positively charged amino acid side chains could play an important role by neutralizing the negative charge barrier thus favouring the approach of the protein to the nucleic acid. As shown by Bedouelle and Winter (1986) three clusters of Arg and Lys residues are involved in the interaction between the tyrosil tRNA synthetase from *Bacillus stearothermophilus* and $tRNA^{Tyr}$. Rop is a rather acidic protein, having only seven (nine including His) positively charged amino acids compared with the 14 Asp or Glu residues. We have modified to Cys two of the three Lys and three of the four Arg residues and we have tested whether any of these changes affected the activity of the protein. The results shown in Figure 6 indicate that none of the five amino acids that have been altered are absolutely essential for function. The alteration of Lys-3 and Lys-6, however, results in a clear decrease of activity. Although the mutation of the three Arg residues has not revealed any change in function it is still conceivable that other positively charged amino acids (apart from Lys-3 and -6) give a small contribution to the stability of the protein−nucleic acid interaction. In other words the information carried by the positive charges could be redundant and its contribution only detected when the total charge (perhaps only in a restricted area of the protein) falls below a certain threshold. This point could be resolved by the construction of multiple mutants where two or more amino acids are altered at the same time.

Other mutants on the lateral surface of the Rop cylinder

Most of the remaining mutants, constructed by altering the solvent exposed side chains in the lateral surface of the Rop cylinder, do not alter the function of the protein. The aromatic ring of Phe-14, however, is indicated by our experiments as playing an important role. Although it has been recently appreciated that aromatic rings can act as hydrogen bond acceptors (Levitt and Perutz, 1988), the side chain of Phe is rather hydrophobic and it is therefore unlikely to contribute to the RNA binding process by specifically recognizing a pattern of complementary hydrogen bond donors and acceptors on the bases. It is tempting to speculate that while side chains near the bend (Asn-27, Glu-28) provide the pattern of hydrogen bonds that permits recognition, the aromatic ring of Phe-14 contributes to stabilize the Rop−RNAI−RNAII complex by stacking between two consecutive bases. In Figure 7 we have summarized our results by depicting in red van der Waal surfaces on those side chains whose alteration renders the protein defective. Blue surfaces identify the side chains that we have modified without altering the activity of the protein. The most striking characteristic, evinced by rotating the Rop dimer, is that the 'essential' amino acids are asymetrically located on the two surfaces of the molecule perpendicular to the 2-fold axis.

We have recently demonstrated that Rop protects the stems of RNAI and RNAII from ribonuclease digestion (Helmer-Citterich *et al.*, 1988). However the low affinity between the two molecules has until now precluded experiments aimed at identifying the nucleotides that make contact with the Rop dimer. This uncertainty still leaves us with too many degrees of freedom and discourages us from attempting any serious docking experiments. The results that we have presented suggest that.

(i) The Rop molecule (its structure and folding mechanism) is rather insensitive to amino acid substitutions and to other alterations as drastic as deletions and insertions. This

property will be discussed in detail elsewhere.

(ii) Together with the aromatic chain of Phe-14, a small number of side chains clustered at the extremities of the Rop cylinder play an important role in the interaction between the two RNAs that underlie plasmid copy number control. These essential side chains all belong to the first helix and are placed on one of the two surfaces of the molecule perpendicular to the 2-fold axis (Figure 7).

Materials and methods

Strains and general techniques

Two bacterial strains were commonly used in this work. They were both derived from 71/18 Δ*lac-pro/F'*[*lacI*^{q} *lacZ*Δ*M15 proAB*$^+$] *supE* (Messing *et al.*, 1977). L76 is the strain used for the red/white test and is 71/18 lysogenized with the λ phage ΦBG36 that contains a transcriptional fusion between the promoter of the primer for initiation of ColE1 replication and the β-galactosidase structural gene. 71/72 was used to test overproduction of the Rop protein and consists of 71/18 transformed with plasmid pCI857, a plasmid compatible with pEX43 containing the temperature sensitive allele of the λ repressor and conferring resistance to the antiobiotic kanamycin. The amber mutants were tested in a *sup*^{o} strain isogenic to L76 called L75.

Microbiological techniques, recombinant DNA, site directed mutagenesis and sequencing were according to standard protocols with minor variations. The oligonucleotide used for mutagenesis and sequencing ranged in length from 16–22 nt.

Construction of pEX43

The expression vector pEX43 was constructed in the following way. First we inserted in the *Acc*I site of pEMBL9 (Dente *et al.*, 1983) the 309-nt long *Hpa*II fragment of pBR322 containing the Rop gene. The plasmid that contained the Rop gene in the same orientation as the α-peptide of pEMBL9 was named pC19. We then inserted the *Eco*RI–*Hae*II fragment (treated with S1) of pLC28 (Remaut *et al.*, 1983) containing the λp_L promoter into the filled in *Hin*dIII site of pC19. In one of the two possible orientations (pEX35) and λ promoter directs the synthesis of Rop mRNA. We then deleted from pEX35 the *Aat*II–*Cla*I fragment immediately preceding the β-lactamase gene to obtain pEX36. The deleted fragment contained a portion of the Rop gene and caused problems in site directed mutagenesis experiments. Finally we obtained pEX43 by inserting into the *Pst*I site a fragment of 57 nt that contains the Φ10 promoter of bacteriophage T7 (Tabor and Richardson, 1985).

Red-white test and copy number assay

To test whether a given plasmid was able to direct the synthesis of an active Rop mutant we transformed it into L76 and then we either plated a suitable dilution to obtain single colonies or we spotted it, together with appropriate controls, on a MacConkey plate (Difco) containing 0.5% lactose and 50 μg/ml of ampicillin. The development of the red colour was monitored after different times (20–48 h) depending on the temperature of the assay.

The relative copy number was evaluated by measuring the ampicillin concentration at which the colony forming ability of a given mutant was decreased by a factor of 10 after plating at 37°C. The plates used in the test (LB medium) contained 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mg/ml of ampicillin. In a typical experiment pEX43, containing the wild type Rop gene, shows a plating efficiency of 0.1 at 2.5 mg/ml.

Carboxylation assay

To overexpress small amounts of isotopically pure Rop we made use of a commericial coupled transcription–translation system (Dupont Nek 038). The synthesized, ^{35}S-labelled, Rop protein was incubated at different temperatures, for different time lengths, in 0.5 M Tris acetate pH 8.7 in the presence of 10 mM iodo-acetic acid. After the carboxylation reaction the sample was made to 100 mM DTT and 6 M urea, heated at 95°C for 5 min and electrophoresed on a 15% polyacrylamide Laemmli gel containing 6 M urea and without SDS. After electrophoresis the signal was intensified by fluorography (Enlighting Dupont) and the dried gel was autoradiographed by exposing to a Kodak X-omat X-ray film.

Crystallographic techniques

The mutant protein Rop Δ[Asp-30–Gln-34] was purified using the standard procedure (Banner *et al.*, 1987). The best crystals were obtained by vapour diffusion using ammonium sulphate as a precipitant. The mutant protein crystallizes in the same space group (C2) as the native protein, yielding large crystals which diffract at high resolution (1.5 Å) and are not particularly sensitive to X-ray irradiation. All crystallographic measurements were made on an Enraf-Nonius CAD-4 diffractometer. The structure was solved by molecular replacement using the refined coordinates of residue 1–25 and 36–56 from wild type Rop as starting model. The quality of the map obtained was sufficient to follow the polypeptide chain along the entire molecule, including the seven C-terminal residues which were disordered in the wild type protein. The structure of this mutant will be described in detail elsewhere.

Acknowledgements

This work was carried out in the framework of the contract no. BAP-0252-D (to G.C.) and BAP-0247-GR (to M.K.) of the Biotechnology action program of the Commission of the European Communities. We wish to thank P.Neuner for oligonucleotide synthesis, D.Kirk and A.Kingswell for technical assistance, and A.Tramontano for generous help with the Insight program. Grazia Bottaro constructed the mutants Asp-32–Trp and Asp-32–Glu.

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Received on October 4, 1988; revised on December 5, 1988

Control of ColE1 DNA Replication: The rop Gene Product Negatively Affects Transcription from the Replication Primer Promoter

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PNAS 1982;79;6313-6317
doi:10.1073/pnas.79.20.6313

Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6313–6317, October 1982
Genetics

Control of ColE1 DNA replication: The *rop* gene product negatively affects transcription from the replication primer promoter

(operon fusion/primer transcription/repressor)

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Communicated by Donald R. Helinski, July 6, 1982

ABSTRACT **A 600-base-pair region essential for ColE1 and pMB1 plasmid replication contains two promoters responsible for the synthesis of two RNA molecules central to copy number control. One promoter directs synthesis of the primer RNA precursor. The second promoter directs the synthesis of a small RNA molecule, RNA1, which acts in *trans* to inhibit processing of the RNA primer precursor. We have fused each promoter to the β-galactosidase structural gene contained in a λ phage. Expression of the RNA1 promoter in lysogens is not influenced by the presence of wild-type pMB1 or ColE1 plasmids residing in the cell. Transcription from the RNA primer promoter, however, is repressed by the product of a *trans*-acting plasmid gene product, which we have designated *rop* (for repressor of primer). The *rop* gene maps downstream from the replication origin in a region that encodes a polypeptide of 63 amino acids whose sequence is completely conserved in pMB1 and ColE1. We propose that this polypeptide is the *rop* gene product and that it regulates plasmid DNA replication by modulating the initiation of transcription of the primer RNA precursor.**

The small multicopy plasmid ColE1 and its close relative pMB1 have been extensively studied as model systems for the investigation of control of initiation of DNA replication (1).

Two regions of the ColE1 genome carry information involved in the control of initiation of DNA replication and plasmid copy number. The first is located in a symmetrically transcribed region approximately 400 nucleotides upstream from the replication origin (2, 3). This region codes on one strand for part of the primer precursor and on the other strand for a small RNA of 108 nucleotides, known as RNA1 (Fig. 1). The larger RNA is processed by RNase H to yield a molecule of 555 nucleotides, which serves as a primer for the initiation of DNA replication *in vitro* (4). The smaller RNA has been shown to inhibit DNA replication by interfering with the processing of the primer by interacting with the complementary structure in the primer precursor (5–7).

The second *trans*-acting regulatory element was mapped by Twigg and Sherratt (8) to a region bounded by two *Hae* II restriction sites 806 and 184 nucleotides downstream from the replication origin of pMB1 and designated the *Hae* II fragment C region. This region is located between the replication origin and the mobilization genes of ColE1 and is not essential for plasmid replication. It is not known whether this second regulatory element participates in the same inhibitory circuit in which RNA1 is involved or whether it controls plasmid copy number by a different mechanism.

Here we show that the *Hae* II fragment C region encodes a *trans*-acting element that inhibits transcription initiated at the primer promoter. We have localized the element to a region that can code for a 63 amino acid polypeptide. The sequence of this polypeptide is conserved in the related plasmids ColE1 and pMB1. We present evidence that this inhibitor controls the initiation of DNA replication by decreasing the amount of primer available for initiation of DNA synthesis.

MATERIALS AND METHODS

Strains, Enzymes, and General Techniques. *Escherichia coli* K-12 71/18, Δ[*lac pro*], F'lac I^qZ ΔM15 pro^+, was the bacterial strain used as a host for the β-galactosidase fusion phages (9). λ phage 132 (10) was obtained from Barbara Meyer.

General microbiological techniques were essentially as described by Miller (11) or by Brenner *et al.* (12). Restriction enzymes and ligase were obtained from V. Pirrotta and H. Cambier. Radiochemicals were purchased from Amersham. Minicell preparation and labeling were done as described by Reeve (13).

Construction of β-Galactosidase–Promoter Fusion Phages. Plasmid DNA (10 μg) digested by restriction endonucleases (*Hae* III, *Alu* I, and *Hpa* II) was ligated to a 7-fold excess of *Hin*dIII linkers. *Hpa* II-digested DNA fragments were made blunt-ended by incubation with DNA polymerase I Klenow subunit (Boehringer Mannheim) in the presence of 0.1 mM dCTP and dGTP.

Restriction fragments from the replication region were purified on a polyacrylamide gel and ligated to *Hin*dIII-digested λ 132 DNA (1:1 molar ratio).

After *in vitro* packaging, phage were recovered by plating on strain 71/18 in 5 ml of 0.7% agarose in L broth. Plates containing 100–1,000 plaques were screened by plaque hybridization (14) using nick-translated pBR322 plasmid as a probe. Purified plaques were further checked by hybridization to different *Hpa* II fragments from the replication region.

The orientation of the DNA fragments inserted into the *Hin*dIII site of phage 132 was determined by recombination with plasmids in which the plasmid is inserted in a known orientation (15). Occasionally, clones harboring DNA fragments that were known to contain an active promoter were identified by directly plating on MacConkey plates and screening for red plaques.

Construction of Lysogens and Assay for β-Galactosidase. Lysogens of 71/18 harboring fusion prophages were constructed by low multiplicity infection of a bacterial culture and selection on plates containing approximately 10^8 λ phage 1052 (*h80 att80 imm21* cI^{ts}). Immune colonies were purified twice in the absence of the selective phage and at least three independent colonies per lysogenization were analyzed for β-galactosidase activity in order to avoid picking lysogens carrying

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Abbreviation: bp, base pair(s).

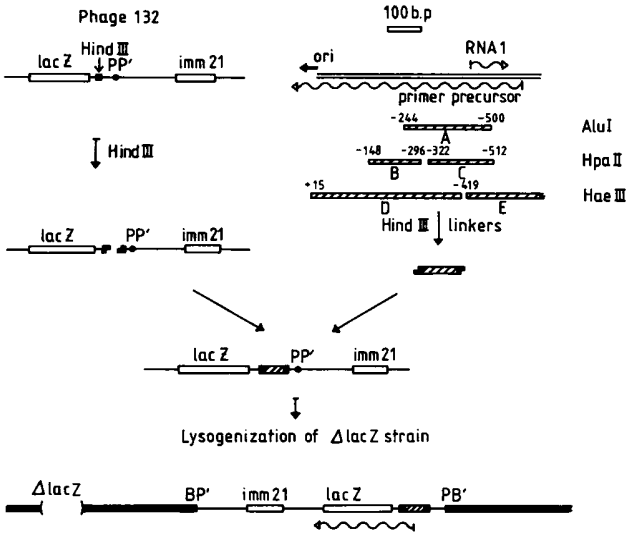


FIG. 1. Fusion of replication promoters to the β-galactosidase gene. The drawing shows a schematic representation of the strategy used to fuse restriction fragments that originate from the pMB1 replication origin to the β-galactosidase structural gene. The bars beneath the transcription map represent the restriction fragments that have been cloned in phage 132. Numbers above the bars refer to the distance in base pairs (bp) of the restriction sites from the replication origin. Phage (P, P') and bacterial (B, B') attachment sites are indicated. Wavy lines represent RNA transcripts.

multiple prophages. Derivatives harboring different plasmids were isolated after $CaCl_2$ treatment and transformation.

Quantitation of β-galactosidase synthesis was essentially as described by Miller (11).

Construction of pDL180. In order to delete the 180-nucleotide DNA fragment between the *Pvu* II and *Acc* I restriction sites immediately downstream from the replication origin, 1 μg of pBR322 DNA was partially digested with *Acc* I. After the ends generated by *Acc* I had been filled in, the plasmid DNA was digested to completion with *Pvu* II. The digested DNA was ligated at low concentration and used to transform 71/18 containing the fusion phage φBG34. The structure of those plasmids that were unable to repress β-galactosidase synthesis in this strain was determined by restriction endonuclease digestion.

DNA Sequence Analysis. The DNA sequence of the *Hae* II fragment C region in the ColE1 derivative pRSF2124 was obtained by the chemical degradation method of Maxam and Gilbert (16).

RESULTS

Phage 132 (Fig. 1) was constructed (10) to facilitate the identification of elements involved in transcriptional regulation. This vector includes a unique *Hin*dIII site preceding a functional β-galactosidase structural gene that lacks its natural promoter. When this vector is inserted into the bacterial chromosome, β-galactosidase expression is dependent on the activity of any upstream promoter sequence inserted at the *Hin*dIII site.

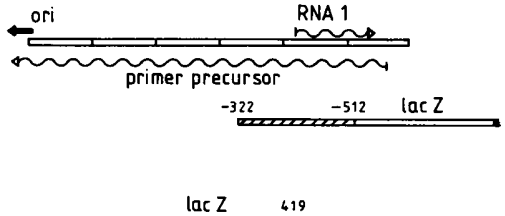
The advantages that a phage system offers for the study of promoter activities are particularly relevant when the promoters considered are involved in the control of plasmid replication functions. A phage system allows the integration of promoter–β-galactosidase fusions in single copy into the bacterial chromosome. As a consequence, promoter activity can be studied in the absence of possible interference by plasmid copy number effects or regulatory elements present in a plasmid vector.

Restriction fragments from the region essential for pMB1 plasmid replication (17) were inserted into the *Hin*dIII site of phage 132 to identify transcription signals that might be important for plasmid replication. Of all the DNA fragments tested, only fragments that contain the promoters for RNA1, P_{RNA1} (Fig. 1, fragments A and C), or for the RNA primer precursor, P_{primer} (Fig. 1, fragment E), were able to promote β-galactosidase synthesis, and they could do this only when they were inserted in the correct orientation.

Fig. 2 shows the relative strengths of the promoters in the replication region, measured as β-galactosidase activity in extracts of bacteria harboring a prophage with the relevant promoter fusion. The strength of the promoters measured *in vivo* is qualitatively consistent with the results obtained *in vitro* (4). The promoter for RNA1 is approximately 10 times stronger than the primer precursor promoter (compare lines A and B, column 1, Fig. 2).

To determine whether plasmid-encoded elements could control transcription starting from P_{RNA1} or P_{primer}, we measured β-galactosidase synthesis directed by these fusion phages in cells harboring the plasmids pMB3 or pBR322. pMB3 is a pMB1 derivative containing the Tn3 transposon (18).

β-Galactosidase synthesis directed by P_{RNA1} is not influenced by a resident pMB1 plasmid derivative (Fig. 2, line A, columns 1, 2, and 3). By contrast, β-galactosidase expression in fusion strains that contain the primer promoter is inhibited by a plasmid-encoded, *trans*-acting element specified both by pMB3 and by its smaller derivative pBR322 (Fig. 2, line B, columns 1, 2, and 3). When the transcriptional activity provided by the



FUSION		β galactosidase activity: no plasmid 1	+pMB3 2	+pBR322 3
φ BG 39	A	450±60	420±60	420±60
φ BG 34	B	65±10	27±5	25±5
132	C	23±5	20±5	20±5

FIG. 2. β-Galactosidase activity in bacterial strains lysogenized with different fusion prophages. β-Galactosidase units are defined as in Miller (11). Each activity is the mean of at least three measurements that did not differ by more than 15%.

Table 1. β-Galactosidase activities in lysogens

Plasmid present	Ref.	Replication origin	β-Galactosidase activity: φ132, no promoter	φBG34, RNA primer pMB1	φBG29, RNA primer ColE1
1. None			23	65	73
2. pBR322	19	pMB1	24	29	27
3. pAT153	8	pMB1		72	71
4. pDL180	This work	pMB1		46	
5. pac162	7	pMB1		34	
6. pac162 *svir11*	7	pMB1		30	
7. pCR1	20	ColE1		24	30
8. pNOP42II	3	ColE1		60	70
9. pDS4156	8	ColK		42	45
10. pDS4152	8	ColK		63	74
11. pJN59	21	CloDF13		45	
12. pNOP1	3	ColE1		26	
13. pUC8	J. Messing	pMB1		68	
14. pUC8-HpaII$_{309}$	This work	pMB1		28	

Lysogens containing no insert promoter (φ132) or the primer promoter from either pMB1 (φBG34) or ColE1 (φBG29) were transformed with a variety of plasmids containing replication origins as listed in the third column. Relevant genetic features of the residing plasmids are described in the text. The data are units of β-galactosidase activity as described by Miller (11) and are the means of at least three measurements that did not differ by more than 15%.

prophage above is considered (Fig. 2, line C), it is apparent that the β-galactosidase activity directed by the primer promoter in the presence of the inhibitor is not significantly above background levels.

Genetic Mapping of the *rop* Gene. The plasmid pac162 *svir11* contains the replication region of pMB1 with a single base pair alteration in the RNA1 coding sequence that reduces the ability of RNA1 to interact with the wild-type target site (7). However, pac162 *svir11* is capable of inhibiting β-galactosidase expression directed by the primer promoter (Table 1, compare lines 1, 5, and 6). Therefore, either RNA1 has two separate functions that can be independently modified or, alternatively, this molecule is not involved in controlling primer transcription. The plasmid pAT153 (8) is a pBR322 derivative in which the DNA fragment corresponding to the ColE1 *Hae* II fragment C has been deleted (Fig. 3). pAT153 does not code for the inhibitory activity (Table 1, line 3). This function can be restored by the insertion of a 576-bp *Sau*3A/*Acc* I fragment located downstream from the replication origin of pMB1 (Fig. 3). Thus, all the information essential for the inhibitory activity must be contained in this 576-bp DNA fragment. The gene that encodes the *trans*-acting negative regulator has been designated *rop* for repressor of primer.

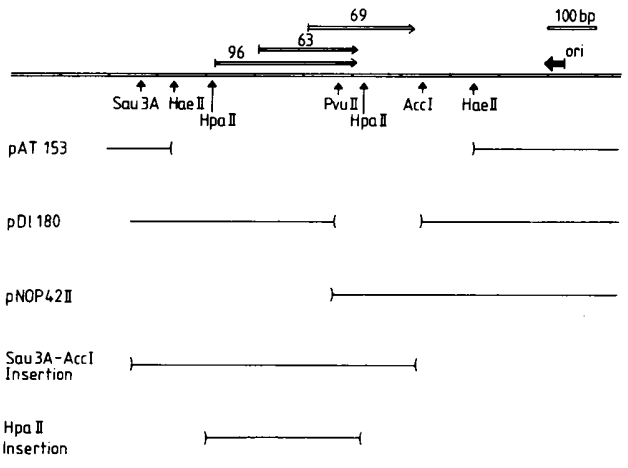


FIG. 3. Translation map of the region approximately 600 bp downstream from the replication origin in the plasmid pBR322 (19). Horizontal arrows represent open reading frames. Figures above the arrows are the lengths of the polypeptides (in amino acids) that could be coded for by the open reading frames. Beneath the translation map the limits of the DNA deletions and insertions that affect *rop* activity are marked by curved vertical lines.

Comparison of *rop* Activity in Different Plasmids. To investigate whether the ColE1 primer promoter was a target for the *rop* function, a phage was constructed that contained the β-galactosidase structural gene under the control of this promoter. This phage, φBG29, contains a *Hae* III fragment from nucleotides 420–834 upstream from the ColE1 origin inserted into the *Hin*dIII site of phage 132 (22). Like φBG34, φBG29 contains the primer promoter but not the RNA1 promoter. Various plasmids were tested for their ability to provide *rop* gene activity as measured by their effects on β-galactosidase synthesis in strains carrying either φBG34 or φBG29 as lysogens (Table 1). It is clear that the primer promoter from ColE1 does not differ appreciably from that of pMB1 in its sensitivity to the *rop* gene activity provided by either ColE1- or pMB1-derived plasmids (Table 1, lines 2 and 7). This result is not unexpected because the DNA sequence of the two promoters is highly conserved (19, 22).

Twigg and Sherratt (8) reported that the ColE1-related plasmid ColK could reduce the copy number of ColE1 derivatives from which the repressor encoded in the ColE1 *Hae* IIC region has been deleted. The ability of ColK to complement the ColE1 deletions was mapped to the vicinity of the ColK *Hae* II B fragment. We measured the *rop* gene activity derived from ColK derivatives that contain or lack the *Hae* IIB region. pDS4156 contains the *Hae* IIB region and shows weak but detectable *rop* activity on both ColE1 and pMB1 primer promoters (Table 1, line 9). pDS4152 has a deletion of the ColK *Hae* II fragment B and lacks *rop* activity (Table 1, line 10). The ColK *rop* gene is less active on ColE1 and pMB1 targets than the *rop* activity from the homologous plasmids (Table 1, compare lines 2 and 7 with line 9). Similarly, the multicopy plasmid CloDF 13 also encodes an inhibitor that is slightly active on the pMB1 target (Table 1, line 11).

Comparison of the DNA Sequence of the *rop* Genes in ColE1 and pMB1. A deletion of ColE1 sequences to the left of the *Pvu* II site in the *Hae* II C fragment (pNOP42II in Fig. 3) abolishes *rop* activity (Table 1, line 8). This result confirms the fact that the ColE1 *rop* gene maps in a position equivalent to that of the pMB1 *rop* gene, as expected (8). Inspection of the pMB1 DNA sequence (19) in the vicinity of the *Pvu* II site reveals the presence of two overlapping open reading frames. The first could encode two polypeptides of 96 and 63 amino acids, depending on which of two possible start codons is utilized (Fig. 4). The 96-amino acid polypeptide would initiate from an ATG codon at position 1,817 in pBR322. The shorter polypeptide would be translated starting from a GTG initiation codon that is preceded by a potentially active ribosome-binding site G-G-A-G-G [boxed in Fig. 4 (23)]. The second open reading frame could encode a very basic polypeptide of 69 amino acids starting at an ATG codon at position 2,010 of pBR322 (Fig. 4).

To investigate whether the amino acid sequences of these three potential polypeptides were conserved in ColE1, we partially determined the sequence of the *Hae* II C fragment of ColE1. The ColE1 plasmid whose sequence was determined was derived from pRSF2124 (24), which contains the Tn3 transposon inserted downstream from the replication origin. Sequence analysis revealed that in pRSF2124 the Tn3 transposon integrated into the ColE1 genome at a position corresponding to that between nucleotides 1,867 and 1,868 of pBR322. This position is 667 bp downstream from the ColE1 origin of replication and interrupts the potential 96-amino acid open reading frame of ColE1 (Fig. 4). However, pNOP1, a pRSF2124 derivative that contains Tn3 inserted in the same position of ColE1 DNA, displays full *rop* gene activity (Table 1, line 12). Consequently, the 96-amino acid open reading frame cannot encode the *rop* gene product.

In Fig. 4 the differences between the pMB1 and ColE1 sequences in the region encoding the 69- and 63-amino acid polypeptides are shown. Three single base deletions and 19 substitutions are present in the ColE1 sequence corresponding to the pMB1 fragment that encodes these polypeptides. Strikingly, each of the 11 substitutions in the sequence that encodes the 63 amino acid polypeptide are third position changes that do not alter the amino acid sequence. By contrast, the 12 substitutions in the potential gene encoding the 69-amino acid polypeptide cause 8 changes in the amino acid sequence. These considerations suggest that the 63-amino acid polypeptide is the more likely candidate for the *rop* function in pMB1 and ColE1.

FIG. 4. Sequence of pBR322 and ColE1 DNAs in the *rop* gene region. Sequence changes in ColE1 in this region relative to pBR322 DNA are shown below the position in ColE1. Single-base deletions not found in ColE1 DNA are denoted by Δ. The location of the Tn3 insertion in pRSF2124 and its derivatives is denoted by Λ. The initiation and termination codons for the 63, 69, and 96 amino acid open reading frames are underlined. The locations of relevant restriction sites are noted. A potential ribosome-binding sequence immediately upstream from the GTG initiation codon of the 63 amino acid polypeptides is boxed.

Identification of the *rop* Gene Product. To assign the *rop* gene activity to a polypeptide encoded in the region immediately downstream from the replication origin, we examined polypeptides synthesized in minicells containing plasmids whose *rop* activity had been measured (Table 1). Minicells containing pBR322 synthesize a prominent polypeptide migrating with an approximate molecular weight of 6,500 (Fig. 5, lane 3, band C). In the absence of other information, however, the resolution of the gel system does not permit discrimination between polypeptides of 63 and 69 amino acids.

The plasmid pDL180 is a deletion derivative of pBR322 lacking the 180-bp *Pvu* II/*Acc* I fragment in the *rop* region (Fig. 3). This deletion removes most of the 69-amino acid encoding sequence but only 13 amino acids from the COOH terminus of the 96/63-amino acid encoding sequence (Fig. 4). In minicells pDL180 encodes a M_r 14,000 band (labeled A, Fig. 5, lane 2) not seen in minicells containing pBR322 (compare lanes 2 and 3, Fig. 5). Inspection of the DNA sequence of pDL180 indicated that an open reading frame of 128 amino acids is generated, resulting from a fusion of the 63-amino acid start codon with in-frame sequences terminating at a TGA codon at position +55 from the replication origin. The expected fusion polypeptide originating from the 63-amino acid start codon corresponds in size to the observed band A in Fig. 5, lane 2. The fusion polypeptide retained 50 amino acids from the NH_2 terminus of the 63-amino acid polypeptide. When tested for *rop* gene activity, pDL180 showed about half the activity of pBR322, suggesting that the fusion polypeptide retains substantial *rop* activity (Table 1, line 4). These results are consistent with the 63-amino acid polypeptide being the *rop* gene product and argue against a role for the 69-amino acid polypeptide.

Greater insight into the relationship between the 69- and 63-amino acid polypeptides and the *rop* gene product was provided by the following cloning experiment. The plasmid pUC8 is a 2,900-bp ampicillin-resistant pBR322 derivative that lacks *rop* activity (Table 1, line 13). Moreover, pUC8 does not direct syn-

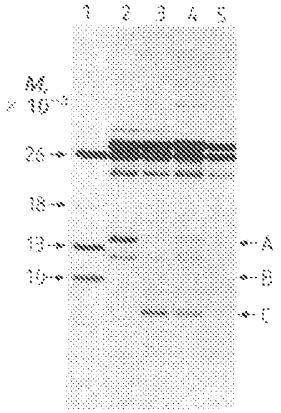


FIG. 5. Analysis of plasmid-encoded polypeptides in minicells. Minicells were isolated and labeled with [^{35}S]methionine as described by Reeve (13). Extracts were analyzed on a sodium dodecyl sulfate/20% polyacrylamide gel and autoradiographed. Lane 1, marker polypeptides consisting of labeled concanavalin A species (Amersham). Corresponding molecular weights are shown at left. Lanes 2–5, minicell extracts from pDL180 (lane 2), pBR322 (lane 3), pUC8-*Hpa*II$_{309}$ (lane 4), and pUC8 (lane 5). A, B, and C refer to the position of the fusion polypeptide from pDL180 and the expected positions of the 96-amino acid polypeptide and the 63-amino acid polypeptide, respectively.

thesis of a M_r 6,500 polypeptide in minicells (Fig. 5, lane 5). We constructed a derivative of pUC8 that contained the 309-bp *Hpa* II fragment between nucleotides 1,811 and 2,120 of pBR322 (shown schematically in Fig. 3; sequence shown in Fig. 4). This *Hpa* II fragment contains the 96- and 63-amino acid reading frames but not the 69-amino acid reading frame. pUC8-*Hpa*II$_{309}$ provides the *rop* gene activity when tested with the pMB1 primer promoter *in vivo* (Table 1, line 14). In minicells, this pUC8 derivative synthesizes a prominent polypeptide that comigrates with that synthesized from pBR322 (Fig. 5, lane 4, band C). Because this derivative cannot synthesize a 69-amino acid polypeptide we conclude that the M_r 6,500 product is 63 amino acids in length and is the product of the *rop* gene.

DISCUSSION

The activity of the promoters for RNA1 and the RNA primer precursor in the plasmids pMB1 and ColE1 were studied by fusion to a β-galactosidase gene with its own promoter deleted. β-Galactosidase, synthesized under the control of the RNA1 promoter, is not affected by any plasmid gene product.

By contrast, we have identified a gene (*rop*) that encodes an inhibitor of RNA primer transcription. When a plasmid carrying an intact *rop* gene was present in cells in which β-galactosidase was expressed under the control of the primer promoter, β-galactosidase synthesis was reduced to background levels.

The *rop* gene is located approximately 600 nucleotides downstream from the replication origin. Twigg and Sherratt (8) have shown that ColE1 and pMB1 plasmids deleted for this region show an increase in copy number. In addition, they showed that the copy number of these plasmids could be returned to wild-type levels by derivatives of the related plasmid ColK, which provides the deleted function in *trans*. We have tested these ColK derivatives for their *rop* activity on the primer promoters of pMB1 and ColE1 and concluded that ColK codes for an inhibitor that is active on the two promoters. The correlation between *rop* activity and control of copy number suggests the involvement of the *rop* gene product in the control of plasmid replication.

This mechanism of control of initiation of DNA replication is not dependent on RNA1, because we have shown that mutations in RNA1 do not affect the functioning of the *rop* gene. Therefore, it is likely that the *rop* control system utilizes a regulatory mechanism independent from that previously characterized (5–7).

It is not clear why ColE1 has two apparently different mechanisms to control the initiation of DNA synthesis. The localization of the *rop* gene in the region essential for plasmid mobilization may mean that the *rop* gene product has a role in transfer replication, possibly through repression of vegetative replication during the conjugation process. Alternatively, replication control in ColE1 may result from the cumulative effects of RNA1 and the *rop* gene product acting to inhibit functional primer formation at two distinct levels: RNA1 affecting primer maturation by interfering with RNase H processing, and the *rop* gene product affecting the frequency of primer transcriptional initiation.

DNA sequence analysis suggests that the *rop* gene product is a 63-amino acid polypeptide whose predicted sequence is entirely conserved in ColE1 and pMB1. The isolation of mutants in this region will be necessary to confirm this prediction.

That the *rop* gene is not involved in plasmid incompatibility is indicated by the fact that plasmids compatible with ColE1 such as ColK specify a *rop* function. The increased copy number of mutants with the *Hae* II C region deleted (8) suggests that the *rop* gene is involved in the control of initiation of DNA replication. The results presented here suggest that the *rop* gene product may modulate plasmid replication by limiting the amount of primer precursor available for RNase H processing.

We thank A. Dunn, V. Pirrotta, and T. Blumenthal for critically reading the manuscript, A. Biais for typing the manuscript, and the photographic department of the European Molecular Biology Laboratory for preparation of the figures. G.C. is grateful to N. Murray and R. M. Lacatena for continuous encouragement and discussions. We thank H. M. Shepard for contributions to the DNA sequence analysis. This work was paritally supported by a National Institutes of Health grant (to B.P.). M.A.M. was supported by a National Institutes of Health predoctoral training grant in molecular biology. B.P. is the recipient of a National Institutes of Health Research Career Development Award (AI00407).

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